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(54) Antiviral agent.

(57) A protein-bond polysaccharide, useful as an antiretroviral agent and particularly in the treatement of AIDS,

(i) possessing a molecular weight of from 50,000 to 3,000,000 as measured by gel permeation chromatography;

(ii) exhibiting a positive color reaction characteristic in the α-naphthol sulfuric acid reaction, the indole sulfuric acid reaction, the anthrone sulfuric acid reaction, the phenol sulfuric acid reaction, the tryptophane sulfuric acid reaction, the Lowry-Folin method and the ninhydrin reaction after hydrochloric acid hydrolysis;

(iii) in which the ratio of the weight of the protein moiety determined by the Lowry-Folin method to the weight of the saccharide moiety determined by the phenol sulfuric acid method is from 40:60 to 70:30;

(iv) showing solubility in Water and insolubility in pyridine, chloroform, benzene, hexane Bnd methanol,

(v) exhibiting a specific rotation, $[\alpha]_D^{25}$ of from -10° to 30°,

(vi) showing a characteristic absorption at 890 cm^{-1} in the infra-red absorption spectrum,

(vii) containing the amino acids aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in an amount of not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and

(viii) containing as saccharides glucose and mannose in an amount of not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being of from 2:1 to 4:1.

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ANTIVIRAL AGENT

The present invention relates to a specific proteinbond polysaccharide derived from fungi belonging to Coriolus of Bacidiomycetes and an antiviral agent, particularly an antiretroviral agent, and more particularly an antiAIDS virus agent (anti-AIDS drug), containing protein-bond polysaccharide as an active ingredient.

More particularly, the present invention relates to a protein-bond polysaccharide possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reactions characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing the weight ratio of protein moiety determined by the Lowry-Folin method to saccharide moiety determined by the phenol sulfuric acid method in the range of 40/60 to 70/30, showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having as an amino acid aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in which the content thereof is not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and having as a saccharide glucose and mannose in which the content thereof is not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being in the range of 2 : 1 to 4 : 1, and to an antiviral agent containing the aforementioned proteinbond polysaccharide as an active ingredient.

As an anti-AIDS drug, azido-3'-deoxythymidine (AZT) which has been already used in actual clinic may be cited. This particular agent is observed to entail a side effect of obstructing the mitosis of normal cells. Though vaccines have been heretofore used as antiviral agents, they are not effective as an anti-AIDS drug.

As protein-bond polysaccharides derived from fungi of Basidiomycetes and process for producing such protein-bond polysaccharides, the followings have been proposed.

As protein-bond polysaccharide possessing a molecular weight in the range of 5,000 to 300,000 as measured by the the ultracentrifugal method, exhibiting a positive color reactions characteristic of saccharides in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction and the tryptophane sulfuric acid reaction, exhibiting a positive color reactions characteristic of peptide bond and amino acid in Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing absorption regions of 0.9 ± 0.1 ppm, 1.2 ± 0.1 ppm, 2.0 ± 0.1 ppm, 4.5 ± 0.1 ppm and 4.7 ± 0.1 ppm and a broad absorption region of 3.0 to 4.4 ppm as measured by proton nuclear magnetic resonance spectrum, having the ratio of saccharide moiety to protein moiety in the range of 55/45 to 95/5 provided that the proton strength of the protein moiety falls in the range of 0.5 to 2.5 ppm and the proton strength of saccharide moiety in the range of 2.5 to 6.0 ppm, having the saccharide moiety composed of β-glucan, showing no absorption attributed to an α-glucan of 4.9 to 6.0 ppm, and having the protein moiety composed of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, tryptophane, phenylalanine, lysine, histidine and arginine; and a process for producing the aforementioned protein-bond polysaccharide by preculturing a seed culture of a basidiomycetous fungus thereby inducing growth of fungal lichen on the surface of a culture medium, homogenizing the fungal lichen with physiological saline solution thereby preparing a seed culture for production culture, cultivating the seed culture by stationary or submerged culture thereby obtaining a mycelium, extracting the mycelium with hot water or an aqueous solvent such as dilute alkaline solution, concentrating the extract after removal of residue therefrom, salting out the concentrate with ammonium sulfate or subjecting to ultrafiltration thereby removing low molecular substances therefrom and obtaining a refined concentrate, adding an amount of ammonium sulfate equivalent to a saturation degree of 25% to the resultant concentrate thereby inducing precipitation, removing the precipitate from the resultant solution, adding an amount of ammonium sulfate equivalent to a saturation degree of 40% to the resultant solution, collecting the precipitate consequently formed in the solution, desalting a solution of the precipitate by dialysis, subjecting the resultant desalted solution to DEAE cellulose column, eluting the adsorbate with an aqueous 1 mol sodium chloride solution, adding an amount of ammonium sulfate equivalent to a saturation degree of 40% to the eluate, collecting the precipitate consequently formed in the resultant reaction solution, and desalting a solution of the precipitate by dialysis [Japanese Patent Publication No. 55-23,271 (1980)].

An antitumor agent suitable for oral administration to a mammal having cancer, said antitumor agent being capable of retarding the growth of said cancer, which comprises an effective amount of a protein-bound polysaccharide obtained by extraction of a mycelium or fruit body of a Coriolus of Polyporaceae of

Basidiomycetes and having a molecular weight within the range of 5,000 to 300,000 as measured with ultracentrifugation and which gives a positive color reactions characteristic of saccharides with α-naphthol-sulfuric acid, indolesulfuric acid, anthrone-sulfuric acid, phenol-sulfuric acid, and tryptophane-sulfuric acid, a positive ninhydrin color reaction characteristic of amino acids after hydrochloric acid hydrolysis and a positive color reaction in the Lowry-Folin test characteristic of peptide linkage, said protein-bound polysaccharide having absorption regions, as measured by proton nuclear magnetic resonance spectra, at 0.9 ± 0.1 ppm, 1.2 ± 0.1 ppm, 2.0 ± 0.1 ppm, 4.5 ± 0.1 ppm and 4.7 ± 0.1 ppm, with broad absorption at 3.0-4.4 ppm, said protein-bound polysaccharide having a polysaccharide portion/protein portion ratio within the range of 55/45 to 95/5 provided that the proton strength of the protein portion is at 0.5-2.5 ppm and that of the polysaccharide portion is at 2.5-6.0 ppm, and said polysaccharide portion being composed of β-glucan, showing no absorption attributed to α-glucan at 4.9-6.0 ppm, said protein portion being composed of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, tryptophane, phenylalanine, lysine, histidine and arginine, said polysaccharide being present in an amount sufficient to retard the growth of cancer (U.S. Patent No. 4271151).

A polysaccharide possessing a molecular weight in the range of 5,000 to 300,000 as measured by the ultracentrifugal method, exhibiting a positive color reactions characteristic of saccharides in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, and tryptophane sulfuric acid reaction having elementary analyses of 43.5 to 45.3% of carbon, 5.7 to 6.7% of hydrogen, and the balance of oxygen, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of 70° to 180° showing a characteristic absorption at 840 cm^{-1} in the infrared absorption spectrum exhibiting absorption regions of 3.7 ± 0.1 ppm, 3.8 ± 0.1 ppm, 5.0 ± 0.1 ppm, and 5.4 ± 0.1 ppm, exhibiting solubility in water and insolubility in chloroform and hexane, and possessing D-glucose as a main component saccharide [Japanese Patent Publication No. 5646,481 (1981)].

A polysaccharide having a molecular weight of from 5,000 to 300,000 as determined by ultracentrifugation, giving color reactions characteristic of saccharides in α-naphthol-sulfuric acid reaction, indole-sulfuric acid reaction, phenol-sulfuric acid reaction, and tryptophane-sulfuric acid reaction, containing 43.5 to 45.3% by weight of carbon, 5.7 to 6.7% by weight of hydrogen and the balance of oxygen and being free of nitrogen, the saccharide units of said polysaccharide being composed principally of D-glucose bonded together entirely by α-linkage and having a structure in which $\rightarrow {}^{4}G^{1} \rightarrow$ is within the range of 3.5 to 8.5, $\rightarrow {}^{3}G^{1} \rightarrow$ is less than 2, $\rightarrow {}^{4}G\,{}^{1}_{6} \rightarrow$ (with the 6 above the 4) is within the range of 0.5 to 2.0, $\rightarrow {}^{4}G\,{}^{1}_{3} \rightarrow$ (with the 3 above the 4) is within the range of 0.1 to 2.5 and $\rightarrow {}^{3}G\,{}^{1}_{6} \rightarrow$ (with the 6 above the 3) is less than 0.8 when the non-reducing endgroup ($G^{1} \rightarrow$) of monosaccharide as determined in methylation-hydrolysis test according to Haworth's method is indexed as 1, the specific rotatory power $[\alpha]_D^{25}$ of said polysaccharide being + 70° to + 180°, said polysaccharide showing a specific absorption at 840 cm^{-1} in its infrared absorption spectrum and showing absorptions at 3.7 ± 0.1, 3.8 ± 0.1, 5.0 ± 0.1, and 5.4 ± 0.1 ppm but not showing any absorptions in the range of 4.4 to 4.9 ppm in its nuclear magnetic resonance spectrum, and said polysaccharide being soluble in water but insoluble in pyridine, chloroform, and hexane, produced by the process comprising the steps of,

extracting mycelia, fruit bodies of a basidiomycetous fungus selected from the group consisting of Coriolus versicolor (Fr.) Quél., Coriolus consors (Berk.) Imaz., Coriolus hirsutus (Fr.) Quél. and Coriolus pargamenus (Fr.) Pat. or mixtures thereof with an aqueous solvent selected from the group consisting of water, an aqueous dilute acid solution, an aqueous 0.005 to 2N solution of potassium- or sodium hydroxide, and an aqueous dilute solution of an organic solvent,

saturating the thus-obtained extract solution with ammonium sulfate after removing the low-molecular weight substances with molecular weight of lower than 5,000 contained therein by ultrafiltration, reverse osmosis or a combination thereof, collecting the resultant precipitate,

dissolving said precipitate in water,

desalting the thus-obtained solution of said precipitate,

passing the thus-desalted solution through a column packed with an ion exchanger, thereby absorbing and removing the nitrogenous substance contained therein,

concentrating the thus-obtained solution, and

drying the thus-obtained concentrate to obtain said polysaccharide (U.S. Patent No. 4614733).

A protein-polysaccharide possessing a molecular weight in the range of 5,000 to 300,000 as measured by the ultracentrifugal method, exhibiting a positive color reactions characteristic of saccharides in α-

naphthol sulfuric acid reaction, indole sulfric acid reaction, and tryptophane sulfuric acid reaction, exhibiting a positive color reactions characteristic of peptide bond and amino acid in Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing absorption regions of 0.9 ± 0.1 ppm, 1.2 ±0.1 ppm, 2.0 ± 0.1 ppm, 4.5 ± 0.1 ppm, 4.7 ± 0.1 ppm, 5.0 ± 0.1 ppm, and 5.4 ± 0.1 ppm and a broad absorption regions of 3.0 to 4.4 ppm as measured in the proton nuclear magnetic resonance spectrum, having the ratio of saccharide moiety to protein moiety in the range of 55/45 to 95/5 provided that the proton strength of the protein moiety falls in the range of 0.5 to 2.5 ppm and the proton strengh of the protein moiety falls in the range of 0.5 to 2.5 ppm and the proton strength of the saccharide moiety in the range of 2.5 to 6.0 ppm, having the saccharide moiety comprising β-D-glucan and α-D-glucan, and a ratio of absorption intensive (α/β) of the absorption attributed to the α-glucan of 4.9 to 6.0 ppm to the absorption attributed to the β-glucan of 4.4 to 4.9 ppm in the range of 50/50 to 10/90, and having the protein moiety composed of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, tryptophane, phenyl alanine, lysine, histidine and arginine; and a process for producing the aforementioned protein-bond polysaccharide comprising preculturing a seed culture of a basidiomycetous fungus thereby inducing growth of a fungal lichen on the surface of a culture medium, homogenizing the fungal lichen with physiological saline solution thereby producing a seed culture for production culture, cultivating the seed culture by stationary or submerged culture thereby obtaining a mycelium, extructing the mycelium with hot water or an aqueous solvent such as dilute alkali solution, concentrating the extract after removal of residue therefrom, salting out the concentrated extract with ammonium sulfate or subjecting to ultrafiltration for removal of low molecular substances and production of a refined concentrate, saturating the resultant concentrate to 40% with ammonium sulfate, removing from the concentrate the precipitate, then saturating the resultant solution to 60% with ammonium sulfate, collecting from the solution the thus formed precipitate, desalting a solution of the separated precipitate by dialysis, subjecting the desalted solution to DEAE-cellulose column, and subsequently eluting the adsorbate with an aqueous 1 mol sodium chloride solution [Japanese Patent Publication No. 57-40,159 (1982)].

A nucleic acid-containing glycoprotein, having a molecular weight of from 5,000 to 300,000 as determined by the ultracentrifugation method; the ratio of the weight of its protein moiety, as determined by Lowry-Folin's method, to the weight of its saccharide moiety, as determined by the phenosulfuric acid method, being from 50:50 to 80:20; the saccharide moiety containing fucose, ribose, arabinose, xylose, mannose, galactose, glucose and glucosamine, and the total weight of said xylose, said mannose and said glucose being more than 85% by weight of the total weight of said saccharides; the protein moiety containing aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine,cysteine, valine, methionine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, tryptophane, ornithine, lysine, histidine and arginine, and the total weight of said aspartic acid, said threonine, said serine, said glutamic acid, said glycine, said alanine, said phenylalanine, said valine, said leucine and said isoleucine being more than 75% by weight of the total weight of all of said amino acids; the amino acid at its N-end being tyrosine, leucine or alanine; the amino acid sequence at its C-end being leucine to phenylalanine to valine, the terminal amino acid being leucine; its elementary composition being from 35.2 to 49.3% of C, from 4.8 to 8.0% of H, from 4.3 to 12.3% of N, from a trace amount to 2.5% of S, from a trace amount to 1.2% of P and the balance being 0, isoelectric point being from pH 2.5 to pH 5.0; nucleic acid containing 0.01 to 0.50% by weight of uracil as a base of nucleic acid; and nucleic acid-containing glycoprotein showing infrared absorption maxima at 3600-3200 cm^{-1}, 1530 cm^{-1} and 1200-1000 cm^{-1}, said nucleic acid-containing glycoprotein being obtained by extracting fruit bodies, mycelia or cultured mycelia of a fungs belonging to Coriolus of Basidiomycetes with hot water or an aqueous 0.01 to 2.0N alkali solution at a temperature of 80 to 100°C for 1 to 8 hours, neutralizing the obtained extract, subjecting the neutralized extract to dialysis and/or ultrafiltration thereby removing a low molecular weight substance having a molecular weight of below 5,000 and fractionally collecting the fractions precipitating under the conditions of the pH of 2.5 to 5.0, of the ion strength of 0.1 to 3.1μ at a temperature of 5 to 25°C (U.S. Patent No. 4663438).

A process for producing a polysaccharide which in the form of the hydrolyzate exhibits a positive color reactions characteristic in Molisch reaction, anthrone sulfuric acid reaction, tryptophane sulfuric acid reaction, cysteine sulfuric acid reaction, aminoguanidine sulfuric acid reaction, Chromotrope sulfuric acid reaction, carbazol-cysteine sulfuric acid reaction, Seliwanoff reaction, Bial test, the aniline hydrochloric acid reaction, Tollens reaction and thioglycolic acid sulfuric acid reaction, and a weakly positive color reaction characteristic in the ninhydrin reaction, and possesses an anticancer activity, which comprises artificially culturing a fungus of genus Coriolus thereby producing a mycelium and extracting the mycelium with water or an aqueous solvent containing a small amount of acid, base, or organic solvent [Japanese Patent Publication No. 51-36,322 (1976)].

A process for the production of a polysaccharide comprising forming a culture medium or a liquid extract of mycelium of a species of fungi belonging to the class Basidiomycetes and selected from the group consisting of Coriolus versicolor, Coriolus conchifer, Coriolus pargamenus, Coriolus hirsutus, Coriolus biformis, Coriolus consors and Coriolus pubescens, separating said polysaccharide from the free proteins, and other impurities contained in said culture medium or liquid extract and purifying said polysaccharide to produce a purified polysaccharide having an acute toxicity (LD50) in mice of more than 20 g/kg for oral administration and more than 100 mg/kg for subcutaneous injection; and

a polysaccharide having a molecular weight within the range of 1.0-1.9 x 100000, as determined by gel filtration, and which, in the form of a hydrolyzate, gives positive ninhydrin, anisaldehyde, molisch, anthrone, tryptophane-sulfonic acid, chromotropic acid-sulfuric acid, carbazole-cysteine-sulfuric acid, aniline-hydrochloric acid, resorcinol-hydrochloric acid, tollens and thioglycol-sulfuric acid reactions but negative ferric chloride and fehling reactions, said polysaccharide being produced by the process comprising forming a culture medium or a liquid extract of mycelium of a species of fungi belonging to the class Basidiomycetes and selected from the group consisting of Coriolus versicolor, Coriolus conchifer, Coriolus pargamenus, Coriolus hirsutus, Coriolus biformis, Coriolus consors and Coriolus puberscens, and separating the polysaccharide from said culture medium or liquid extract (U.S. Patent No. 4051314).

A method for producing a nitrogen-containing polysaccharide, comprising extracting a fungus belonging to Coriolus of Basidiomycetes with an aqueous 0.01N to 2N alkali solution and subjecting the resultant extract to ultrafiltration or reverse osmosis thereby removing from the extract low molecular substances of molecular weights not exceeding 5,000 [Japanese Patent Publication No. 56-14274 (1981)].

In recent years, various viral diseases such as B-type hepatitis, adult T-cell leukemia, and AIDS (Acquired Immunodeficiency Syndrome) have been the subjects of scientific topics.

Particularly, a series of acquired immunodeficiency syndromes popularly referred to as "AIDS" have been attracting interest as a serious disease fatal to the patients. It has been already established by researches that the contraction of this disease occurs when the human T_4 lymphocytic cells adsorb HIV (human immunodeficiency virus), one species of the retrovirus, and the propagation of the disease follows when the other lymphatic cells are affected by the virus. Generally, the viral diseases have been heretofore coped with by preventive inoculation of relevant vaccines. As the result, the smallpox has been exterminated, and the yellow fever and the polimyelitis (infantile paralysis) have been attached.

However, the vaccination is not effective in curing such diseases as AIDS which pose the problem of persistent infection or latent infection. In the light of the nature of the pellicle of HIV, it is considered difficult to develop a vaccine which is effective in attaching the HIV. In the circumstances, the development of a safe anti-AIDS drug which manifests an outstanding effect in curing the disease has been desired.

AZT has already found utility as an anti-AIDS drug. Unfortunately, however, this agent is observed to entail a side effect of obstructing the mitosis of normal cells. Therefore, the development of a safe anti-AIDS drug is desired.

One of the present inventors has already found a number of protein-bond polysaccharides having an activity as biological response modifiers (BRM) on the immune system. Particularly, one species of the protein-bond polysaccharides extracted from a fungus belonging to Coriolus of Basidiomycetes has already been sold as an anti tumor drug under Trademark of "Krestin". This drug shows very low toxicity and can be administered for a long time without any fear for disturbing the intestinal microbe. Further, it is an extremely safe substance because it has no adverse effect on the nature of variation or the reaction of allergy and, therefore, has no possibility of inducing deformation or allergy on a healthy person. Since the protein-bond polysaccharides extracted from fungi belonging to Coriolus of Basidiomycetes which are natural substances and since they contain complicate compounds which are formed with numerous protein-bond polysaccharides, the active substances of the extracts, which are effective in curing AIDS [activity of obstructing the adsorption of HIV on the human lymphatic cells and obstructing the activity of RTxase (reverse transcriptase) which is an enzyme essential for the propagation of HIV] have not yet been fully elucidated.

As a result of a continuously diligent inventors' study with a view to elucidating the behavior of the aforementioned active substances, it has been found that a protein-bond polysaccharide obtained by culturing a fungus of genus Coriolus thereby inducing growth of a mycelium or fruit body, extracting the mycelium or fruit body with hot water or an aqueous alkaline solution, salting out the extract with saturated ammonium sulfate, after redissolving the resultant precipitate, desalting the thus obtained solution by dialysis salting out the resultant solution with an amount of ammonium sulfate equivalent to a saturation degree of 25%, desalting a solution of the resultant precipitate by dialysis, subjecting the resultant solution to DEAE-anion-exchange cellulose column treatment, eluting the adsorbed fraction with an aqueous sodium chloride solution, and desalting the eluate by dialysis, possesses an antiviral activity. The present invention

has been attained based on the finding mentioned above.

In a first aspect of the present invention, there is provided a protein-bond polysaccharide possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reactions characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing the ratio of the weight of protein moiety determined by the Lowry-Folin method to the weight of saccharide moiety determined by the phenol sulfuric acid method in the range of 40/60 to 70/30, showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having as an amino acid aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in which the content thereof is not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and having as a saccharide glucose and mannose in which the content thereof is not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being in the range of 2 : 1 to 4 : 1.

In a second aspect of the present invention, there is provided a protein-bond polysaccharide possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reactions characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing the ratio of the weight of protein moiety determined by the Lowry-Folin method to the weight of saccharide moiety determined by the phenol sulfuric acid method in the range of 40/60 to 70/30, showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having as an amino acid aspartic acid, thereonine, serine, glutamic acid, glycine, alanine, vallne and leucine in which the content thereof is not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and having as a saccharide glucose and mannose in which the content thereof is not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being in the range of 2 : 1 to 4 : 1, which said protein-bond polysaccharide is obtained by desalting a solution of the precipitate produced by salting out an extract of a fungus of Coriolus of Basidiomycetes with saturated ammonium sulfate, (1) salting out the resultant solution with ammonium sulfate equivalent to a saturation degree of 25%, desalting a solution of the obtained precipitate, subjecting the thus desalted solution to a DEAE-ion-exchange cellulose column, then eluting the adsorbed substances with an aqueous sodium chloride solution, and desalting the eluate, or (2) subjecting the thus desalted solution to DEAE-ion-exchange cellulose column, eluting the adsorbed substances with an aqueous sodium chloride solution, desalting the eluate, then salting out the resultant solution with ammonium sulfate equivalent to a saturation degree of 25% and desalting a solution of the obtained precipitate.

In a third aspect of the present invention, there is provided an antiviral agent, comprising as an active ingredient an effective amount of a protein-bond polysaccharide possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reactions characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing the ratio of the weight of protein moiety determined by the Lowry-Folin method to the weight of saccharide moiety determined by the phenol sulfuric acid method in the range of 40/60 to 70/30, showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having as an amino acid aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in which the content thereof is not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and having as a saccharide glucose and mannose in which the content thereof is not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being in the range of 2 : 1 to 4 : 1, and a pharmaceutically acceptable carrier.

In a fourth aspect of the present invention, there is provided an antiretovial agent, comprising as an active ingredient an effective amount of a protein-bond polysaccharide possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reaction characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and

ninhydrin reaction after hydrochloric acid hydrolysis, possessing the ratio of the weight of protein moiety determined by the Lowry-Folin method to the weight of saccharide moiety determined by the phenol sulfuric acid method in the range of 40/60 to 70/30, showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having as an amino acid aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in which the content thereof is not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and having as a saccharide glucose and mannose in which the content thereof is not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being in the range of 2 : 1 to 4 : 1, and a pharmaceutically acceptable carrier.

In a fifth aspect of the present invention, there is provided an anti-AIDS drug, comprising as an active ingredient an effective amount of a protein-bond polysaccharide possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reactions characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, possessing the ratio of the weight of protein moiety determined by the Lowry-Folin method to the weight of saccharide moiety determined by the phenol sulfuric acid method in the range of 40/60 to 70/30, showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having as an amino acid aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in which the content thereof is not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and having as a saccharide glucose and mannose in which the content thereof is not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being in the range of 2 : 1 to 4 : 1, and a pharmaceutically acceptable carrier.

Brief Description of the Drawings:

Fig. 1 is an infrared absorption spectrum of the substance obtained in Example 1 and Fig. 2 and NMR spectrum of the same substance.

The protein-bond polysaccharide of the present invention (hereinafter referred to as the present substance) is characterized by possessing a molecular weight in the range of 50,000 to 3,000,000 as measured by gel permeation chromatography, exhibiting a positive color reactions characteristic in α-naphthol sulfuric acid reaction, indole sulfuric acid reaction, anthrone sulfuric acid reaction, phenol sulfuric acid reaction, tryptophane sulfuric acid reaction, Lowry-Folin method and ninhydrin reaction after hydrochloric acid hydrolysis, having the ratio of the weight of the protein moiety determined by the Lowry-Folin method to the weight of the saccharide moiety determined by the phenon sulfuric acid method in the range of 40/60 to 70/30, exhibiting solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol, exhibiting a specific rotation, $[\alpha]_D^{25}$, in the range of -10° to 30°, showing a characteristic absorption at 890 cm^{-1} in the infrared absorption spectrum, having not less than 70% by weight based on the protein moiety, of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, and leucine, having not less than 75% by weight based on the saccharide moiety, of glucose and mannose, and having the ratio of glucose to mannose in the range of 2 : 1 to 4 : 1.

(1) Preparation of the present substance

The present substance is obtained by culturing a fungus of Coriolus of Basidiomycetes, for example, Coriolus versicolor (Fr.) Quél., Coriolus hirsutus (Fr.) Quél., Coriolus pargamenus (Fr.) Pat. and Coriolus consors (Berk.) Imaz., thereby inducing growth of a mycelium or fruit body, extracting the mycelium or fruit body with hot water or an aqueous alkaline solution, salting out the resultant extract with saturated ammonium sulfate, after redissolving the resultant precipitate, desalting the thus obtained solution by dialysis, (i) salting out the resultant solution with ammonium sulfate equivalent to a saturation degree of 25%, desalting a solution of the resultant precipitate by dialysis, subjecting the resultant solution to DEAE-ion-exchange cellulose column thereby adsorbing a fraction thereon, then eluting the adsorbed fraction with an aqueous sodium chloride solution, and desalting the eluate by dialysis, or (ii) subjecting the resultant

solution to DEAE-ion-exchange cellulose column, eluting the adsorbed fraction with an aqueous sodium chloride solution, desalting the eluate by dialysis, salting out the resultant solution with ammonium sulfate equivalent to a saturation degree of 25%, redissolving the resultant precipitate, and then desalting the thus obtained solution by dialysis.

(2) Physical and chemical properties

① Molecular weight

The molecular weight of the present substance is in the range of 50,000 to 3,000,000 and the average molecular weight thereof is in the range of 110,000 to 300,000 as measured by the gel permeation chromatography.

② Color reaction

When the present substance is dissolved in water for color reaction, the following results are obtained.

Table 1

Color reaction	Color	Result
α-Naphthol sulfuric acid reaction (Molisch reaction)	Purple	Glycide
Indole sulfuric acid reaction	Brown	Glycide
Anthrone sulfuric acid reaction	Green	Glycide
Phenol Sulfuric acid reaction	Brown	Glycide
Tryptophane sulfuric acid reaction	Purple	Glycide
Lowry-Folin method	Blue	Peptide bond, tyrosine, tryptophane, and cysteine
Ninhydrin reaction after hydrochloric acid hydrolysis (6-N HCl, 110° C, 20 hrs)	Purplish blue	α-Amino acid

From the foregoing results of the color reactions, it is clear that the present substance contains saccharides and proteins.

③ Solubility

The present substance is soluble in water and substantially insoluble in methanol, pyridine, chloroform, benzene and hexane.

④ pH value

A solution of 1 g of the present substance in 100 ml of water shows a pH value of 6.0 to 7.5, indicating that the substance is nearly neutral.

⑤Specific rotation

The specific rotation (%), $[\alpha]_D^{25}$, calculated from the optical determined of an aqueous solution containing the present substance in a concentration of 0.10% is in the range of -10° to + 30°, implying that the present substance has β-glycan as the main component thereof.

⑥ Elementary analyses

By elementary analysis, the present substance is found to be composed of 5 to 10% of nitrogen, 35 to 50% of carbon and 5 to 7% of hydrogen.

⑦ The weight ratio of the content of the saccharide moiety of the present substance determined by the phenol sulfuric acid method to the content of the protein moiety of the present substance determined by the Lowry-Folin method falls in the range of 60/40 to 30/70, preferably 54/46 to 35/65.

(3) Structural characteristic

The characteristic of the protein moiety and the characteristic of the saccharide moiety of the present substance are as follows.

① Characteristic of protein moiety

The protein moiety of the present substance is hydrolyzed and then analyzed with an amino acid analyzer in accordance with the conventional process. An amino acid composition is shown below in Table 2.

Table 2

Amino acid	Weight (%)
Aspartic acid	10 ~ 19
Threonine	4 ~ 10
Serine	3 ~ 11
Glutamic acid	10 ~ 18
Glycine	6 ~ 9
Alanine	6 ~ 13
Valine	5 ~ 11
Leucine	6 ~ 8
Proline	Trace ~ 8
Cystine	Trace
Methionine	Trace ~ 4
Isoleucine	3 ~ 5
Tyrosine	Trace ~ 3
Phenylalanine	3 ~ 6
Tryptophane	Trace ~ 2
Lysine	1 ~ 4
Histidine	Trace ~ 2
Arginine	1 ~ 4
(Ammonia)	1 ~ 6

From the Table given above, it is clearly noted that the protein moiety as an active component of the present substance contains 18 amino acids and has acidic amino acids and neutral amino acids as major components and basic amino acids as minor component. It is further noted that not less than 70% of the total amount of the amino acids is composed of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, and leucine.

② Structure of saccharide moiety

The test of the saccharide moiety of the present substance for saccharide composition is carried out by subjecting 10 mg of a sample to methanolysis at 100°C for 16 hours with 3% methanol hydrochloride, then subjecting the product after methanolysis to trimethylsilyl treatment by the conventional process, and analyzing the thus obtained product by gas chromatography. The results of this test indicate that a weight

ratio of glucose to mannose is in the range of 2 : 1 to 4 : 1, and principal component saccharides of the saccharide moiety are glucose and mannose, and not less than 75% of the saccharide moiety is composed of glucose and mannose. Such saccharides as galactose, xylose, fucose, and glucosamine are also contained therein.

The saccharide moiety of the present substance, for the purpose of confirming the D-L distinction of glucose which is one of the main component saccharides thereof, is hydrolyzed and the glucose crystals isolated from the hydrolyzate are assayed, and as a result the glucose crystals are found to possess a melting point of 143 to 145°C, thus showing no fall of melting point and identifying themselves to be D-glucose.

③ Bonding characteristic of component saccharides of the saccharide moiety

The bonding position of glycoside is determined as follows. The monosaccharides isolated from the saccharide moiety by the periodic acid oxidation method and the Smith degradation method are confirmed to possess the bonds, G^1-, $-^4G^1$-, $-^4G\,\frac{1}{3}$-,

$-^4G\,\frac{1}{6}$-, $-^3G^1$-, $-^3G\,\frac{1}{6}$-,

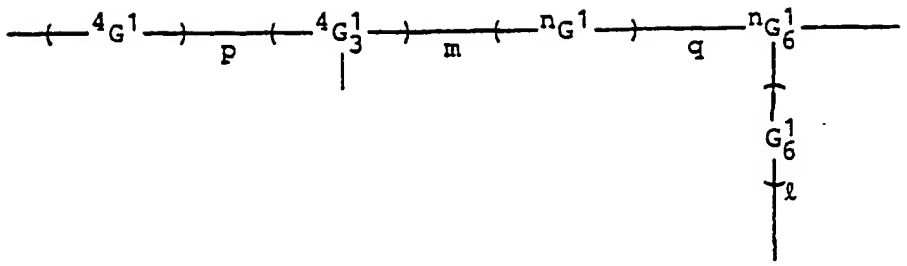
and $-^5G^1$-. The percentage of these bonds is determined by methylating hydrolysis in accordance with the Haworth method. The identification is performed as follows. The saccharides formed by the hydrolysis of the methylation product are identified as alditol acetate and methyl glycoside by gas chromatography. Further the individual hydrolyzates are identified by being isolated by column liquid chromatography and then directly crystallized or converted into crystalline derivatives. The molar ratios are calculated from the ratios of areas formed on the gas chromatograph of alditol acetate, based on $\rightarrow{}^4G^1\rightarrow + \rightarrow{}^3G\,\frac{1}{6} = 1$.

Table 3

Hydrolyzate of methylated saccharide	Bond	Molar ratio
2,3,6-Tri-O-methyl-G	$\rightarrow{}^4G^1\rightarrow$	0 ∿ 10
2,3,4-Tri-O-methyl-G	$\rightarrow{}^6G^1\rightarrow$	0 ∿ 1
2,3-Di-O-methyl-G	$\rightarrow{}^4G^1_{\uparrow 6}\rightarrow$	0 ∿ 1
2,6-Di-O-methyl-G	$\rightarrow{}^4G^1_{\uparrow 3}\rightarrow$	0 ∿ 2
2,4,6-Tri-O-methyl-G	$\rightarrow{}^3G^1\rightarrow$	0 ∿ 5
2,4-Di-O-methyl-G	$\rightarrow{}^3G^1_{\uparrow 6}\rightarrow$	0 ∿ 1

It is clearly shown from the table given above that the saccharide moiety of the present substance is formed mainly with the β-1,4 linkage. The saccharide moiety nevertheless contains the β-1,3 linkage. Thus, it is found to possess a structure highly abounding with branches.

The saccharide moiety, therefore, is concluded to possess a structure represented by the following general formula (I):



(wherein G represents a monosaccharide, p is in the range of 0 to 10, q is in the range of 0 to 5, m is in the range of 0 to 2, n is 3 or 4, and ℓ is in the range of 0 to 1).

The bonds between the saccharide moiety and the protein moiety of the present substance mainly contain those bonds which are formed via the medium of glucosamine. The structure of the present substance is specifically such that either of the hydroxyl groups bonded to the carbon atoms at the positions of C3, C4, and C6 of glucosamine is bonded to the saccharide moiety, and the hydroxyl group at the C1 position and the protein moiety are bonded to each other through the N-glucoside bond.

④ Infrared spectrum

The infrared absorption spectrum of the present substance obtained by the KBr tablet method is as shown in Fig. 1. The broad absorption region of 3,600 to 3,200 cm^{-1} in Fig. 1 is presumed to originate from ›OH's hydrogen bonded to varying degrees. This presumption is based on the observation that this absorption either diminishes or disappears when the hydroxyl groups of the saccharide moiety of the sample is O-methylated. The absorption of 1,700 to 1,600 cm^{-1} is presumed to arise from the deformation vibration of $-NH_2$ and the absorption of 1,530 cm^{-1} is presumed to arise from that of -NH respectively, and they are considered to originate from the protein moiety of the sample. The broad absorption of 1,200 to 1,000 cm^{-1} is presumed to arise from the asymmetrical stretching vibration of the pyranose ring C-O-C bond in the saccharide moiety. While a specific absorption attributed to the β-configuration of glucose of the saccharide moiety is recognized at 890 cm^{-1}, virtually no specific absorption attributed to the α-configuration is recognized at 840 cm^{-1}. The aforementioned infrared absorption spectrum is not recognized to reveal any significant error with respect to the present substance.

⑤Proton nuclear magnetic resonance (NMR)

The NMR of the present substance is obtained at 100 MHz using heavy water as a solvent and sodium 2,2-dimethyl-2-silanopentane-3-sulfonate (D.S.S.) as an internal standard. In Fig. 2 which shows the spectrum consequently obtained, since the absorption at 4.5 ppm is known to be an absorption attributed to the β-(1-4) and β-(1-6) in the methine proton at the 1 position and the absorption at 4.7 ppm to be an absorption attributed to the β-(1-4) and β-(1-6) in the methine proton at the 1 position, the ratio of β-(1-4) and β-(1-6) to β-(1-3) can be determined. Since the structure of the present substance contains branches, precise elucidation of the structure must rely inevitably on the methylation method. The absorption at 5.0 ppm is attributed to the α-(1 → 6) and absorption at 5.4 ppm is attributed to the α-(1 → 4) and α-(1 → 3).

Based on D.S.S., the present substance possesses absorption at 0.9 ± 0.1 ppm, 1.2 ± 0.1 ppm, 2.0 ± 0.1 ppm, 4.5 ± 0.1 ppm, and 4.7 ± 0.1 ppm, no absorption in the region of 4.9 to 6.0 ppm, and a broad absorption in the region of 3.0 to 4.4 pom. The absorptions in the region of 0.5 to 2.5 ppm are attributed to the side-chain proton of the protein moiety and those in the zone of 2.5 to 4.7 ppm are attributed to the proton of the saccharide moiety.

As described above, the present substance is a novel β-glycopeptide obtained from a protein-bond polysaccharide derived from a fungus of Coriolus of Basidiomycetes, and containing no α-glycan as determined by NMR and no nucleic acid as determined by high speed liquid chromatography using ultraviolet light at 254 nm.

(4) Acute toxicity

The present substance is highly safe for living organisms because it manifests very low toxicity and induces virtually no side effect.

The present substance is tested for acute toxicity by the following method.

Mice of the ICR-JCL strain 4 to 5 weeks in age and 21 to 24 g in weight, and rats of the Donryu rat of 4 to 5 weeks in age and 100 to 150 g in weight are used. The present substance is administered to the animals intravenously, subcutaneously, intraperitoneally, and orally. The animals to which the present substance is administered as diluted with physiological saline solution are kept under observation as to general symptom, fatality and body weight. At the end of the period of observation, the animals are sacrificed for anatomical examination.

As shown in Table 4, the present substance administered at the highest allowable dosage kills none of the animals. In fact, the present substance is extremely safe for living organisms so as to render the calculation of LD_{50} value virtually impossible.

The test results establish that the present substance manifests very low acute toxicity and constitutes itself a safe drug.

Table 4

Kind of animal	Manner of administration	LD_{50} (mg/kg)	
		Female	Male
Mouse	Intravenously	> 1300	> 1300
	Subcutaneously	> 5000	> 5000
	Intraperitoneally	> 5000	> 5000
	Orally	>20000	>20000
Rat	Intravenously	> 600	> 600
	Subcutaneously	> 5000	> 5000
	Intraperitoneally	> 5000	> 5000
	Orally	>20000	>20000

(5) Test for antiviral activity

It has been known that generally a virus is reproduced through a path which comprises deposition of the virus on a target cell, injection of the nucleic acid of the virus into the cell, and subsequent integration of the nucleic acid into the genome of the cell. Particularly in the case of a retrovirus, the path for the reproduction requires to include a step in which RNA, the nucleic acid derived from the virus is transferred into DNA by the action of a RT xase before the nucleic acid is integrated into the genome of the cell.

The inventors have found that the present substance obstructs the deposition of HIV on the human lymphatic cell and the subsequent infection of disease and further obstructs the activity of RT xase. When the present substance is tested for the effect on HIV by a procedure which comprises treating HIV with the present substance used in a concentration of 50μg/ml (the present substance used in Examples 1 to 4) at 0°C for 2 hours, washing the treated HIV, adding the washed HIV to MT-4 cells, incubating the resultant mixture for 3 days, and subsequently taking count of the HIV antigen-positive cells, it is found that virtually all the HIV antigen-positive cells are no longer present, indicating that the present substance is highly effective in obstructing the deposition of HIV on the human lymphatic cells. When the present substance is tested for possible effect upon RT xase activity using the whole messenger RNA of rat liver as a mold, it is demonstrated that the present substance strongly inhibits RT xase activity even at a concentration of 5 μg/ml.

When the present substance is used as an antiviral drug, it can be formulated in any desired from. The administration of the present substance may be effected in any desired manner.

When the present substance is prepared in the form of tablets, pellets, powder, or capsules for oral deministration, the composition contained therein may suitably incorporate therein such additives as binder, enveloper, excipient, lubricant, disinegrator, and wetting agent as generally adopted pharmaceutically. When

the present substance is used in the form of a solution for oral administration, it may be prepared as internal lotion, shake mixture, suspension, emulsion, or syrup. Otherwise, it may be prepared in the form of a dry product which is redissolved immediately prior to use. Such a liquid preparation may incorporate therein any of the additives and preserves in common use. When the present substance is prepared in the form of a solution for administration by injection, the composition of this solution may incorporate therein any of various additives such as stabilizer, buffer solution, preserving agent, and isotonic agent. It may be offered in unit-dose ampoules or in dispensing vials. The aforementioned composition may be in the form of aqueous solution, suspension, solution, or emulsion in oily or aqueous vehicle. The active component may be in the form of powder which, prior to actual use, is redissolved with a suitable vehicle such as sterilized water containing no pyrogenic substance.

The present substance is administered orally or parenterally to men and beasts. The oral administration embraces the form of administration which is effected by placing the preparation under the patient's tongue. The parenteral administration embraces the form of administration which is effected by subcutaneous, intramuscular, or intravenous injection or by instillation. The dosage of the antiviral agent of the present invention is affected by the discrimination between beasts and men as subjects of treatment, the age and individuality of a particular subject for treatment, and the condition of disease. There are times when the dosage must deviate from the range of to be specified below. Generally, for oral administration to a human subject, the daily dose is in the range of 0.1 to 1,000 mg, preferably 1 to 100 mg, per kg of body weight, to be taken as undivided or divided into two or three portions.

The present substance is effective in inhibiting viral contamination, particularly obstructing contamination of a retrovirus possessing a reverse transferase, and depressing AIDS which is caused by HIV infection.

Further, the present substance has a dual effect of obstructing the deposition of HIV on the human lymphatic cells and the subsequent infection and impeding RT xase activity.

The AZT which has already found utility as an antiAIDS drug is observed to manifest a side effect of obstructing the mitosis of even normal cells. In contrast, the present substance manifests extremely low acute toxicity, shows virtually no side effect on normal cells, and proves to be a safe drug. It is a useful antiviral agent because it manifests an action of obstructing infection of virus, particularly a retrovirus. To be specific, the present substance is effective in preventing the infection of a virus, particularly the infection of a retrovirus, especially in depressing AIDS. Even when the present substance is used as an antiviral agent in combination with other agents such as AZT, the effect manifested by the present substance is not impaired. Thus, the combined use of the present substance with other medicine proves to be an effective measure.

Now, the present invention will be described more specifically below with reference to working examples. It should be noted, however, that this invention is not limited to these examples.

Examples 1 to 4:

Strains, CM 101 of Coriolus versicolor (Fr.) Quél [Fermentation Research Institute Deposit (FERM) P No. 2412 (corresponding to ATCC 20547); Examples 1 and 2] , CM 102 of Coriolus versicolor (Fr.) Quél [FERM-P 2413 (corresponding to ATCC 20548); Example 3], and CM 103 of Coriolus versicolor (Fr.) Quél [FERM-P 2414 (corresponding to ATCC 20549); Example 4], were each inoculated to a 200-ml Erlenmeyer flask containing 30 ml of a culture medium using 5% of glucose, 0.2% of peptone, 0.3% of yeast extract, 0.1% of KH_2PO_4, and 0.01% of $MgSO_4 \cdot 7H_2O$ and subjected to static culture at a temperature in the range of 25° to 27° C for 10 days. The fungal lichen consequently grown on the surface of culture medium was homogenized with physiological saline solution to prepare a seed culture. Then, in a 1.0-liter culture jar containing 200 ml of the same culture medium as described above, the aforementioned seed culture was inoculated and cultured therein at 25° to 27° C for 25 days to produce a mycelium of the relevant strain. The amount of the mycelium consequently obtained was 4 to 4.3 g/jar in the case of CM 101, 2.0 to 2.5 g/jar in the case of CM 102, and 2.7 to 3.2 g/jar in the case of CM 103.

Then, 100 g of mycelium of each strain was extracted with 3 liters of distilled water at 98° C for 3 hours while stirring. At the end of the extraction, the extract and the residue were separated from each other. by centrifugal separator. This treatment of extraction to the thus separated residue was repeated. The extracts from the successive treatments were gathered and centrifuged for separation of the residue. Then, the resultant filtrate was concentrated.

The filtrate was treated with an aqueous solution of ammonium sulfate saturated to 100% to produce a precipitate. Then, the precipitate was redissolved in water and the resultant solution was desalted by dialysis using a cellulose membrane. The desalted solution was treated with an aqueous 25% ammonium

sulfate solution. The precipitate consequently obtained was redissolved and desalted by dialysis. The desalted solution was subjected to adsorption using a DEAE-ion-exchange cellulose column. The adsorbed substances were eluted with an aqueous sodium chloride solution (1 mol). The eluate was desalted by ultrafiltration, then concentrated, and spray dried to obtain an objective substance of the present invention. The extraction method used in Examples 2 to 4 was performed in the same manner as the procedure described above, excepting that an aqueous 1/10-N caustic soda solution was used in the reextraction of residue instead of water and the pH value of the extracts was adjusted after completion of the extraction.

The infrared absorption spectrum of the substance obtained by the KBr tablet method as indicated in Table 5 was shown in Fig. 1. In this spectrum, the absorption of νOH in 2,600 to 3,200 cm^{-1}, the deformation vibration of NH_2 in 1,700 to 1,600 cm^{-1}, the deformation vibration of NH at 1,530 cm^{-1}, the broad absorption region in 1,200 to 1,000 cm^{-1} attributed to the vibration of the pyranose ring of the saccharide moiety in the C-O-C ring, and the specific absorption at 890 cm^{-1} attributed to β-linkage of saccharide moiety were visible but the absorption at 840 cm^{-1} attributed to α-linkage was not clearly visible.

Since the infrared absorption spectra obtained of the four samples (Examples 1 to 4) were different very little, that of the sample of Example 1 is shown as a representative.

The NMR determination was performed by using D.S.S. as an internal standard and heavy water as a solvent. Since the NMR spectra obtained of the four samples (Examples 1 to 4) were different very little, that of the sample of Example 1 was shown as a representative.

By the gel permeation chromatography using a sephalose column, the molecular weight was found to fall in the range of 50,000 to 3,000,000 and the average molecular weight in the range of 110,000 to 300,000.

The analysis of amino acids was carried out by the steps of mixing 10 mg of a sample with 4 ml of an aqueous 6N hydrochloric acid solution, freezing the resultant mixture with dry acid acetone, then sealing a tube containing the frozen mixture under a vacuum, heating the contents of the sealed tube at 110° C for 24 hours to effect hydrolysis thereof, drying the resultant hydrolyzate, dissolving the dry hydrolyzate in 30 to 40 ml of a citrate buffer of pH 2.2, and analyzing the resultant solution with an amino acid analyzer.

The test for specific rotation was carried out by measuring the optical rotation (α) of an aqueous solution containing a sample in a concentration of 0.10 % with the D line (589 μm) of sodium in a 5-cm cell and calculating the specific rotation, $[\alpha]_D^{25}$, from the value of the optical rotation (α).

The analysis of the saccharide for component monosaccharides was carried out by placing 3 mg of a sample in a glass ampoule 5 mm in diameter, subjecting the sample to methanolysis with 1.0 ml of a 3% methanolic hydrogen chloride solution at 100° C for 16 hours, neutralizing the hydrochloric acid consequently formed with silver carbonate at room temperature, separating the product of neutralization by filtration, distilling the filtrate to dryness, dissolving the concentrated filtrate with 0.5 ml of dry pyridine, adding 0.2 ml of hexamethyl disilazane and 0.3 ml of trimethyl chlorosilane to the formed solution and allowing the resultant mixture to stand at rest at room temperature for 30 minutes to effect trimethylsilylation, thereafter dissolving the resultant product in chloroform, washing the resultant solution with cold water for removal of excess reagents, dehydrating the washed solution, evaporating the filtrate to dryness, dissolving the residue of the distillation in carbon tetrachloride, and analyzing the resultant solution by gas chromatography.

The mode of the bond of saccharides was determined by the Haworth method. To be more specific, this determination was carried out by dissolving 2 g of a sample in 10 ml of an aqueous 1-N NaOH solution, keeping the resultant solution vigorously stirred under a current of nitrogen gas at 40° to 50° C and, at the same time, adding 20 ml of dimethyl sulfuric acid and 40 ml of an aqueous 30% sodium hydroxide solution dropwise thereto over a period of several hours, allowing the resultant mixture to stand at rest overnight, treating the resultant solution with the same amount of methylation reagent, neutralizing the resultant reaction solution, dialyzing the product of neutralization with running water, vacuum-concentrating the dialyzate, then repeating the aforementioned methylation three times, again neutralizing and dialyzing the product of methylation with running water, then distillating the dialyzate to dryness under a vacuum, dissolving the residue of the distillation in 20 ml of a chloroform-methanol (10 : 1) mixture, adding a petroleum ether-ether (1 : 1) mixture to the resultant solution for precipitation of the methylation product, then hydrolyzing about 20 mg of the methylation product with an aqueous 1-N sulfuric acid solution at 100° C for 16 hours, and introducing the hydrolyzate into alditol acetate by the conventional method to obtain a gas chromatogram. The molar ratios of the component monosaccharides were found from the peak areas on the gas chromatogram.

The degradated products mentioned above were identified on respective gas chromatograms using standard products. Then, hydrolyzates were isolated mutually by column liquid chromatography and were

identified as directly crystallized or converted into crystalline derivatives.

Further, the color reactions, the elementary analysis, the acute toxicity (LD_{50}), the pH value, and the solubility were invariably measured by the conventional methods. The measurements whose procedures have been specifically described above were carried out by following these procedures.

By high speed liquid chromatography using ultraviolet light at 254 nm, nucleic acid of the four samples (Examples 1 - 4) was not found.

The properties and the structural characteristics of the present substance determined as described above are collectively shown in the accompanying tables.

The degree with which the present substance obstructs the reverse transferase activity specifically manifested by the retrovirus was determined by the following method.

In 10 ml of sterilized distilled water, 100 μg of a freeze dried sample of the present substance was dissolved (concentration 10 μg/ml).

An Eppendorf tube having an inner volume of 1.5 ml was charged with 1 μl of 20 mM D.T.T. (dithiothreitol; Sigma Corp), 5 μl of an enzyme reaction solution 5 times as high in concentration (250 mM Tris-HCl (pH 8.3)-250 mM KCl-40 mM MgC_{12}), 1 μl of 3d NTP solution (1 mM dATP-1mM dGTp-1 mM dTTp; Sigma Corp), 1 μl of messenger-RNA (taken from normal rat liver: 1 μg/μl), 0.5 μl of RNase Inhibitor (16 units/μl; Takara Shuzo K. K.), and 1 μl of [α-^{32}P] dcTp (about 800 ci/m.mol, 10 μCi/μl; Amasham Japan K. K.) and was placed in a water bath at 37° C.

After 5 minutes' standing of the reaction tube in the bath, 12.5 μl of the present substance prepared in advance in a concentration of 10 μg/ml was added to the reaction tube and 1 μl of RT xase (derived from Rous Associated virus, 7 units/μl; Takara Shuzo K.K.) was further added thereto. The reaction solution was diluted to a final volume of 25 μl and kept at 37° C to induce reaction.

After the reaction continued for one hour, 5 μl of the reaction solution was allowed to permeate a DEAE paper 2 cm x 2 cm (Toyo Roshi K.K.). The wet paper was air dried. The dry filter paper was shaken as immersed in 10 ml per paper of an aqueous 0.5M Na_2HPO_4 solution to wash off the portion of [α-^{32}P] dCTp which had escaped being used in the DNA synthesis. This procedure was performed five times at intervals of 5 minutes.

Thereafter, the aforementioned DEAE filter paper was placed in a glass vial containing 10 ml of liquid scintillation cocktail (Amasham Japan K.K.) and a radioactivity for one minute was counted by a scintillation counter (Aroka K.K.).

The ratio of inhibition of RTxase activity (%) was calculated on the following formula.

$$\text{Ratio of inhibition of RTxase activity (\%)} = \frac{C_O - C_S}{C_O} \times 100$$

(wherein C_O represents the radioactivity in the absence of the present substance and C_S for the radioactivity in the presence of the present substance

The results are shown in Table 5.

The inhibition of the deposition of HIV (AIDS virus) on human lymphocyte effected by the present substance was tested by the following method (The whole procedure was carried out under sterilized conditions).

A test tube containing 1 ml of a HIV suspension and 1 ml of a solution of the substance (100 μg/ml) was left standing at rest in ice. After the test tube stood in the ice for two hours, 1 ml of the virus suspension was left to be adsorbed on a cell strain derived from human lymphocyte, MT-4 [Jpn. J. Cancer Res. (Gann). 28, 219-229 (1982)] at an infection degree (M.O.I.) ≒ 2. The resultant mixture was centrifuged (2,000 rpm for 10 minutes). The supernatant was discarded and the sedimentod MT-4 cells were floated in RPMI 1640 (Gibco Laboratories, NY) containing 20% FCS in a cell concentration of 2 x 10^5/ml.

On a 96-hole plate, the aforementioned MT-4 cell suspension was dispensed in a unit volume of 100 μl and incubated under the conditions of 5% CO_2 and 37° C. On the third day of the incubation, the sample suspensions were analyzed by the indirect fluorescent antibody method to determine the HIV infectious deposition and the non-deposited cell.

To be more specific, the MT-4 cells were solidified by treatment with methanol and were left reacting with the serum of an anti-HIV infected patient at 37° C. After 30 minutes of the reaction, the cells were washed with PBS and then left reacting at 37° C with fluoresceine isothiocyanate-associated rabbit antihuman IgG (immune globulin).

Under a fluorescent microscope, 500 MT-4 cells were observed to take count of fluorescent positive

cells as HIV-adsorbed cells and fluorescent negative cells as HIV-nonadsorbed cells. The ratio of inhibition of HIV deposition (%) was calculated based on the following formula using the results of the microscopic observation.

$$\text{Ratio of inhibition of HIV deposition (\%)} = \frac{(\text{Number of HIV-nonadsorbed cells}) \times 100}{(\text{Number of HIV-adsorbed cells}) + (\text{Number of HIV-nonadsorbed cells})}$$

The results are shown in Table 5.

Comparative Experiment 1:

Krestin was tested for ratio of inhibition of RTxase activity and ratio of inhibition of HIV deposition under the same conditions as in Examples 1 to 4. The ratios consequently found were both below 10%.

Example 5:

Capsules were obtained by packing hard capsules, No. 0, with 330 mg of the substance obtained in Example 1, with a pressure type automatic packing machine.

Table 5

Example No.	1	2	3	4
Extraction method	Hot water	Aqueous alkaline solution	Aqueous alkaline solution	Aqueous alkaline solution
Molecular weight ($\times 10^4$)	5 - 200	5 - 100	5 - 100	5 - 100
Average molecular weight ($\times 10^4$)	18	12	13	15
Color reaction (saccharide)				
α-Naphthol sulfuric acid reaction	Purple	Purple	Purple	Purple
Indole sulfuric acid reaction	Brown	Brown	Brown	Brown
Anthrone sulfuric acid reaction	Green	Green	Green	Green
Phenol sulfuric acid reaction	Brown	Brown	Brown	Brown
Tryptophane sulfuric acid reaction	Purple	Purple	Purple	Purple
Coloration reaction (protein) Lowry-Folin method	Blue	Blue	Blue	Blue
Ninhydrin reaction (6N-HCl, 20 hr) after hydrochloric acid hydrolysis	Purplish blue	Purplish blue	Purplish blue	Purplish blue

Example No.	1	2	3	4
Specific rotation $[\alpha]_D^{25}$ (°)	-8	+27	+13	+2
pH	6.6	7.2	7.4	7.0
Absorption position (ppm) in NMR spectrum				
0.9 ± 0.1	Yes	Yes	Yes	Yes
1.2 ± 0.1	Yes	Yes	Yes	Yes
2.0 ± 0.1	Yes	Yes	Yes	Yes
4.5 ± 0.1	Yes	Yes	Yes	Yes
4.7 ± 0.1	Yes	Yes	Yes	Yes
5.0 ± 0.1	No	No	No	No
5.4 ± 0.4	No	No	No	No
3.0 ∿ 4.4	Yes	Yes	Yes	Yes
Infrared absorption spectrum				
3600 - 3200 cm^{-1}	Yes	Yes	Yes	Yes
1600 cm^{-1}	Yes	Yes	Yes	Yes
1530 cm^{-1}	Yes	Yes	Yes	Yes
1200 - 1000 cm^{-1}	Yes	Yes	Yes	Yes
890 cm^{-1}	Yes	Yes	Yes	Yes
840 cm^{-1}	No	No	No	No

Example No.	1	2	3	4
Amount of saccharide (% by weight)	52.5	42.7	48.0	51.0
Protein (% by weight)	47.5	57.3	52.0	49.0
Analysis of saccharides (mode of linkage)				
$\rightarrow{}^{1}_{G}{}^{4}\rightarrow$	10	4	2	4
$\rightarrow{}^{1}_{G}{}^{3}\rightarrow$	0.5	1.5	trace	2.0
$\rightarrow{}^{1}_{G}{}^{4}_{\uparrow 6}\rightarrow$	0.7	1.0	0.7	0.6
$\rightarrow{}^{1}_{G}{}^{6}\rightarrow$	0.9	0.7	0.4	0.8
$\rightarrow{}^{1}_{G}{}^{4}_{\uparrow 3}\rightarrow$	1.0	0.8	0.6	0.9
$\rightarrow{}^{1}_{G}{}^{3}_{\uparrow 6}\rightarrow$	0.3	trace	0.3	0.4
Saccharides				
Glucose	71.3	60.7	69.0	60.5
Mannose	18.8	24.3	23.0	27.5
Galactose	3.8	4.8	3.8	5.4
Xylose	2.0	5.0	2.0	3.5
Fucose	3.2	4.0	1.1	1.5
Glucosamine	0.9	1.2	1.1	1.6

Example No.	1	2	3	4
Ratio of component monosaccharides				
Glucose/mannose	3.8	2.5	3.0	2.2
Nitrogen content (%)	7.1	9.2	8.5	7.5
Carbon content (%)	45.1	46.2	39.1	41.4
Hydrogen content (%)	5.6	6.5	5.9	6.0
Amino acid				
Aspartic acid	14.0	15.5	14.9	15.5
Threonine	8.0	7.0	8.4	7.0
Serine	7.6	6.5	9.7	7.8
Glutamic acid	12.7	16.8	15.5	16.9
Proline	trace	2.0	trace	1.0
Glycine	7.3	7.1	8.5	7.7
Alanine	10.0	10.3	10.4	11.0
Cysteine	trace	trace	trace	trace
Valine	8.5	6.9	7.8	6.0
Methionine	2.3	1.6	1.6	1.9
Isoleucine	4.8	4.1	3.1	4.2
Leucine	6.5	6.9	7.0	6.6
Tyrosin	0.3	1.1	trace	1.5
Phenylalanine	4.4	3.6	3.3	3.0

Example No.	1	2	3	4
Tryptophane	1.7	1.0	2.0	trace
Lysine	3.0	2.2	2.0	2.8
Histidine	1.8	1.4	trace	1.2
Alginine	2.7	3.0	2.4	2.2
(Ammonia)	4.4	3.0	3.4	3.7
Total of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, and leucine	74.6	77.0	82.2	78.5
Acute toxicity for mouse (LD_{50}, mg/kg)				
Intravenous, male	1300<	1300<	1300<	1300<
female	1300<	1300<	1300<	1300<
Subcutaneous, male	5000<	5000<	5000<	5000<
female	5000<	5000<	5000<	5000<
Intraperitoneal, male	5000<	5000<	5000<	5000<
female	5000<	5000<	5000<	5000<
Oral, male	20000<	20000<	20000<	20000<
female	20000<	20000<	20000<	20000<

Example No.	1	2	3	4
Acute toxicity for rat (LD_{50}, mg/kg)				
Intravenous, male	600<	600<	600<	600<
female	600<	600<	600<	600<
Subcutaneous, male	5000<	5000<	5000<	5000<
female	5000<	5000<	5000<	5000<
Intraperitoneal, male	5000<	5000<	5000<	5000<
female	5000<	5000<	5000<	5000<
Oral, male	20000<	20000<	20000<	20000<
female	20000<	20000<	20000<	20000<
Ratio of inhibition of RTxase activity*	+++	+++	+++	+++
Ratio of inhibition of HIV deposition *	+++	+++	+++	+++

* ±: <10%, +: 11 - 30%, ++: 31 - 70%, +++: >71%

Claims

1. A protein-bond polysaccharide

(i) possessing a molecular weight of from 50,000 to 3,000,000 as measured by gel permeation chromatography;

(ii) exhibiting a positive color reaction characteristic in the α-naphthol sulfuric acid reaction, the indole sulfuric acid reaction, the anthrone sulfuric acid reaction, the phenol sulfuric acid reaction, the tryptophane sulfuric acid reaction, the Lowry-Folin method and the ninhydrin reaction after hydrochloric acid hydrolysis;

(iii) in which the ratio of the weight of the protein moiety determined by the Lowry-Folin method to the weight of the saccharide moiety determined by the phenol sulfuric acid method is from 40:60 to 70:30;

(iv) showing solubility in water and insolubility in pyridine, chloroform, benzene, hexane and methanol,

(v) exhibiting a specific rotation, $[\alpha]_D^{25}$, of from -10° to 30°,

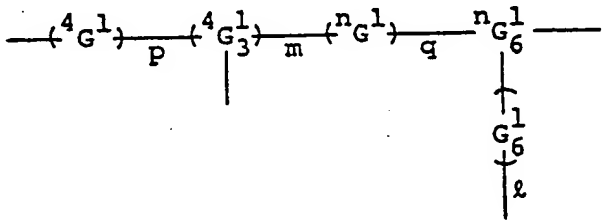
(vi) showing a characteristic absorption at 890 cm^{-1} in the infra-red absorption spectrum,

(vii) containing the amino acids aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine and leucine in an amount of not less than 70% by weight of the total weight of the amino acids of the protein moiety thereof, and

(viii) containing as saccharides glucose and mannose in an amount of not less than 75% by weight of the total weight of the saccharides of the saccharide moiety thereof, the ratio of glucose to mannose being of from 2:1 to 4:1.

2. A polysaccharide according to claim 1, which possesses an average molecular weight of from 110,000 to 300,000 as measured by gel permeation chromatography.

3. A polysaccharide according to claim 1 or 2, wherein the saccharide moiety is represented by the following structural formula (I):



wherein G represents a monosaccharide, p is from 0 to 10, q is from 0 to 5, m from 0 to 2, n is 3 or 4, and 1 is 0 or 1.

4. A polysaccharide according to any one of the preceding claims, wherein the saccharide moiety is bonded to the protein moiety via a glucosamine residue.

5. A polysaccharide according to any one of the preceding claims, for use as an antiretroviral agent.

6. A polysaccharide according to claim 5 for use in the treatment of AIDS.

7. A process for the preparation of a polysaccharide as defined in claim 1, which process comprises culturing a fungus belonging to Coriolus of Basidiomycetes, extracting the mycelium or fruit bodies with water or an aqueous alkaline solution and isolating the said polysaccharide from the extract.

8. A process according to claim 7, wherein the fungus is strain CM 101, CM 102 or CM 103 of Coriolus versicolor (Fr.) Quel.

9. A process according to claim 7 or 8, wherein a solution of the precipitate obtained by salting out the said extract with saturated ammonium sulfate is desalted and the said polysaccharide is obtained by: (1) salting out the resultant solution with ammonium sulfate of a saturation degree of 25%, desalting a solution of the obtained precipitate, introducing the desalted solution onto a DEAE-ion- exchange cellulose column, eluting the adsorbate with an aqueous sodium chloride solution and desalting the eluate or (2) introducing the thus desalted solution onto a DEAE-ion-exchange cellulose column, eluting the adsorbate with an aqueous sodium chloride sodium, deslating the eluate, salting out the resultant solution with ammonium sulfate of a saturation degree of 25% and desalting a solution of the obtained precipitate.

10. A pharmaceutical composition comprising, as an active ingredient, a protein-bond polysaccharide as defined in claim 1 and a pharmaceutically acceptable carrier.

Fig.1

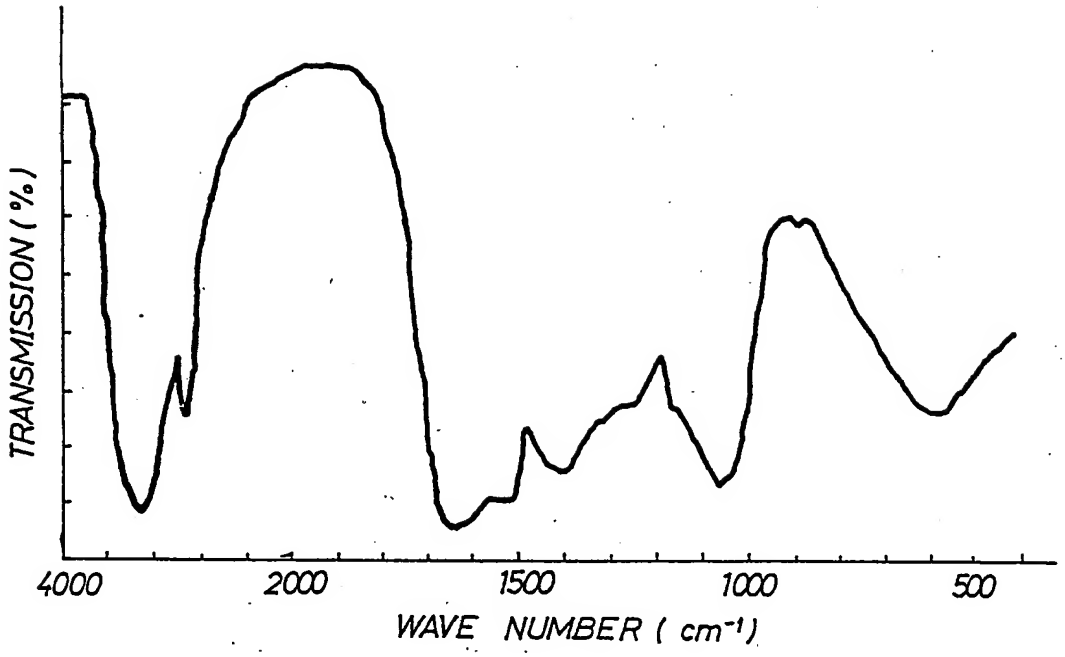
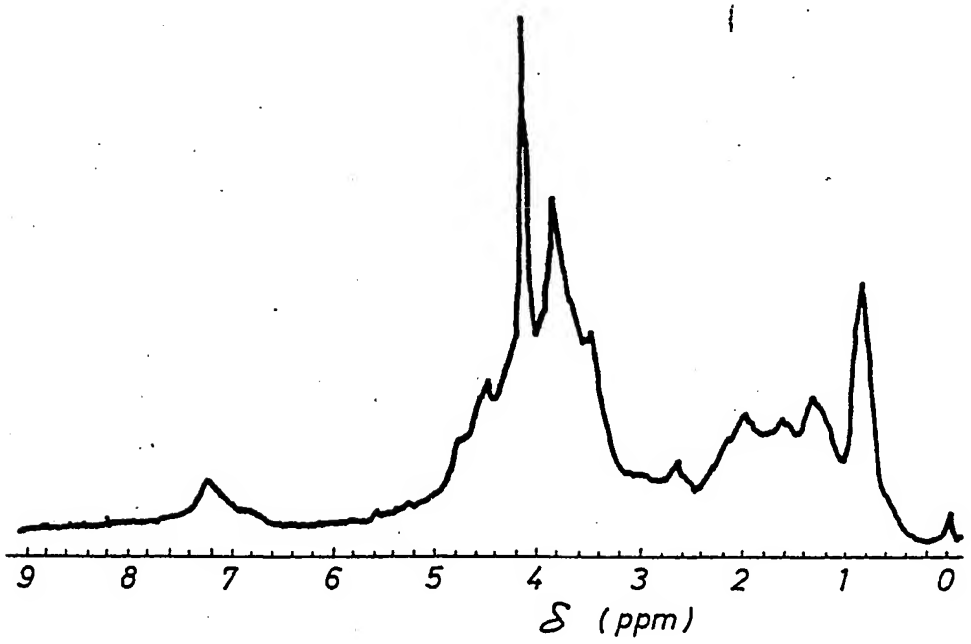

TRANSMISSION (%)
4000
2000
1500
1000
500
WAVE NUMBER (cm⁻¹)

Fig.2


9
8
7
6
5
4
3
2
1
0
δ (ppm)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 30 1602

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,X	EP-A-0 058 093 (KUREHA KAGAKU KOGYO K.K.) * Abstract; page 11, lines 10-18; page 24, lines 30-34; table 5 *	1,7,10	C 07 K 15/14 C 12 P 21/00 C 08 B 37/00 A 61 K 37/02
Y		9	
D,Y	US-A-4 051 314 (KUREHA KAGAKU KOGYO K.K.) * Column 2, lines 20-27; example 25 *	9	
A	CHEMICAL ABSTRACTS, vol. 96, no. 11, 15th March 1982, page 467, abstract no. 84105f, Columbus, Ohio, US; & JP-A-81 112 902 (WAKAMOTO PHARMACEUTICAL CO., LTD) 05-09-1981 * Abstract *	1	
A	CHEMICAL ABSTRACTS, vol. 108, no. 15, 11th April 1988, page 345, abstract no. 127418w, Columbus, Ohio, US; K. HIROSE et al.: "A biological response modifier, PSK, inhibits revese transcriptase in vitro", & BIOCHEM. BIOPHYS. RES. COMMUN., 1987, 149(2), 562-7 * Abstract *	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 07 K C 12 P C 08 B A 61 K
A	FR-A-2 405 298 (LABORATOIRES CASSENNE) * Page 2, lines 9,10; claims 1-3 *	1	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21-04-1989	SOMERVILLE F.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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